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Ligand-dependent contribution of RXRβ to cholesterol homeostasis in Sertoli cells

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We show that mice expressing retinoid X receptor β (RXRβ) impaired in its transcriptional activation function AF-2 (*Rxrb*^{af20} mutation) do not display the spermatid release defects observed in RXRβ-null mutants, indicating that the role of RXRβ in spermatid release is ligand-independent. In contrast, like RXRβ-null mutants, *Rxrb*^{af20} mice accumulate cholesteryl esters in Sertoli cells (SCs) due to reduced ABCA1 transporter-mediated cholesterol efflux. We provide genetic and molecular evidence that cholesterol homeostasis in SCs does not require PPARα and β, but depends upon the TIF2 coactivator and RXRβ/LXRβ heterodimers, in which RXRβ AF-2 is transcriptionally active. Our results also indicate that RXRβ may be activated by a ligand distinct from 9-*cis* retinoic acid.

Keywords: ABC transporters; fatty degeneration; LXR; retinoic acid; spermatogenesis

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INTRODUCTION

Retinoid X receptors (RXRα, β and γ) heterodimerize with numerous nuclear receptors, which include retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs) and liver oxysterol receptors (LXRs), and display a ligand-dependent transcriptional activity that requires the integrity of the activation function 2 (AF-2) core, contained within α-helix 12 (Chambon, 1996; Perlmann & Evans, 1997; Lu *et al*, 2001). In nonpermissive heterodimers, RXRs are transcriptionally inactive, unless their partners are liganded, whereas in permissive heterodimers RXRs can be transcriptionally active, irrespective of the presence of their partner's ligand (Vivat *et al*, 1997). Thus, permissive heterodimerization may integrate two hormonal pathways within a single functional unit. Genetic evidence indicates that the highly pleiotropic developmental effects of retinoic acid are mediated by nonpermissive RXRα/RARα, β and γ heterodimers, in which the AF-2 of RXRα may either be dispensable or required (Mascrez *et al*, 1998, 2001; Matt *et al*, 2003). In contrast, inactivation of RXRβ yields testis-restricted defects absent in vitamin A deficiency or in RAR-null mutants (Kastner *et al*, 1996). To gain further insights into the testicular functions of RXRβ and to identify its heterodimerization partner, we generated a mouse line in which the AF-2 of RXRβ is lacking (*Rxrb*^{af20} mutation), and compared its testicular phenotype with that of mice carrying null mutations of either candidate partners or possible RXR/LXR targets.

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RESULTS AND DISCUSSION

RXRβ AF-2 is dispensable for spermatid release control

The AF-2 of RXRβ was inactivated by homologous recombination in mouse embryonic stem cells, using an *Rxrb* targeting vector in which the sequence coding α-helix 12 was deleted (see supplementary information online). Northern blot analysis indicated that expression of the truncated mRNA in *Rxrb*$^{af2/af2}$ mice (hereafter designated as *Rxrb*^{af20}) was identical to that of RXRβ mRNA in wild-type (WT) littermates (data not shown). *Rxrb*^{af20} mutants, collected from *Rxrb*$^{+/af2}$ intercrosses, were born at mendelian ratio (among 571 littermates, 142 (25%) were WT, 298 (52%) were heterozygotes and 131 (23%) were homozygotes). Their viability, followed over more than a year, was similar to that of WT mice.

In contrast to *Rxrb*$^{-/-}$ males, which are sterile due to scarce and abnormal spermatozoa (Kastner *et al*, 1996), *Rxrb*^{af20} males were fertile (tested up to 12 months) and did not display spermiogenesis defects as their spermatozoa ultrastructure was normal (three mutants were examined; data not shown). Degradation of retained mature (i.e. step 16) spermatids by Sertoli cells (SCs) is a sensitive index of spermiation (spermatid release) failure (Gehin *et al*, 2002), which represents the first hallmark of the *Rxrb*$^{-/-}$ phenotype, and contributes to oligozoospermia (Kastner *et al*, 1996). In *Rxrb*$^{-/-}$ mutants ($n=3$), the seminiferous tubules contained degraded mature spermatids at stages IX–XI (Fig 1D,F), and residual nuclear fragments at all the other stages (e.g. Fig 1B). In contrast, in *Rxrb*^{af20} mutants ($n=5$), the tubules did not display

mature spermatid degradation (Fig 1A,C,E), indicating that RXRβ AF-2 is not required for spermiation. Therefore, assuming that deletion of the AF-2 helix 12 is equivalent to the absence of an agonistic ligand (see Mascrez *et al*, 1998), an unliganded RXRβ can fully support male reproduction.

RXRβ AF-2 deletion results in testis fatty degeneration

The second hallmark of the *Rxrb*$^{-/-}$ phenotype is a progressive lipid accumulation in SCs (Kastner *et al*, 1996). Large lipid droplets were detected in the seminiferous tubules of all ($n=15$) *Rxrb*^{af20} mutants (Figs 1H,I and 2B) before completion of puberty (Fig 1M), and markedly enlarged with age (compare Fig 1I,M). Using electron microscopy (three mutants were examined), these droplets were localized within SC cytoplasms (data not shown). With respect to their early appearance, localization and enlargement with ageing, the *Rxrb*^{af20} droplets were similar to those detected in *Rxrb*$^{-/-}$ testes (Kastner *et al*, 1996), demonstrating that RXRβ AF-2 is indispensable to control SC lipid stores.

A majority of seminiferous tubule cross-sections from 12-month-old *Rxrb*^{af20} mutants ($n=5$) showed normal germ cell associations (Fig 1H). The other tubules displayed a degeneration manifested by a loss of cell populations resulting from the detachment of healthy immature germ cells (Fig 1I), rather than from increased apoptosis (data not shown). In older mutants ($n=3$), this degeneration yielded tubular ghosts devoid of all epithelial cells, including SCs, and moreover filled with lipids that were often calcified (Fig 1J,K). A similar fatty degeneration was reported for *Rxrb*$^{-/-}$ testes (Kastner *et al*, 1996), whereas testes from 24-month-old WT were histologically normal (data not shown).

It has been proposed that the small lipid droplets normally observed in WT seminiferous tubules (Figs 1G,L and 2A) arise through the SC phagocytic activity that is normally responsible for elimination of apoptotic germ cells and cyclic degradation of lipid-rich residual bodies originating from mature spermatids. It was also suggested that their enlargement in testis degenerations could be secondary to increased phagocytosis of dying germ cells (Russell *et al*, 1990). In both *Rxrb*$^{-/-}$ and *Rxrb*^{af20} testes, SC lipid overload reflects a cell-autonomous defect, as it precedes degeneration by several months and is not associated with an

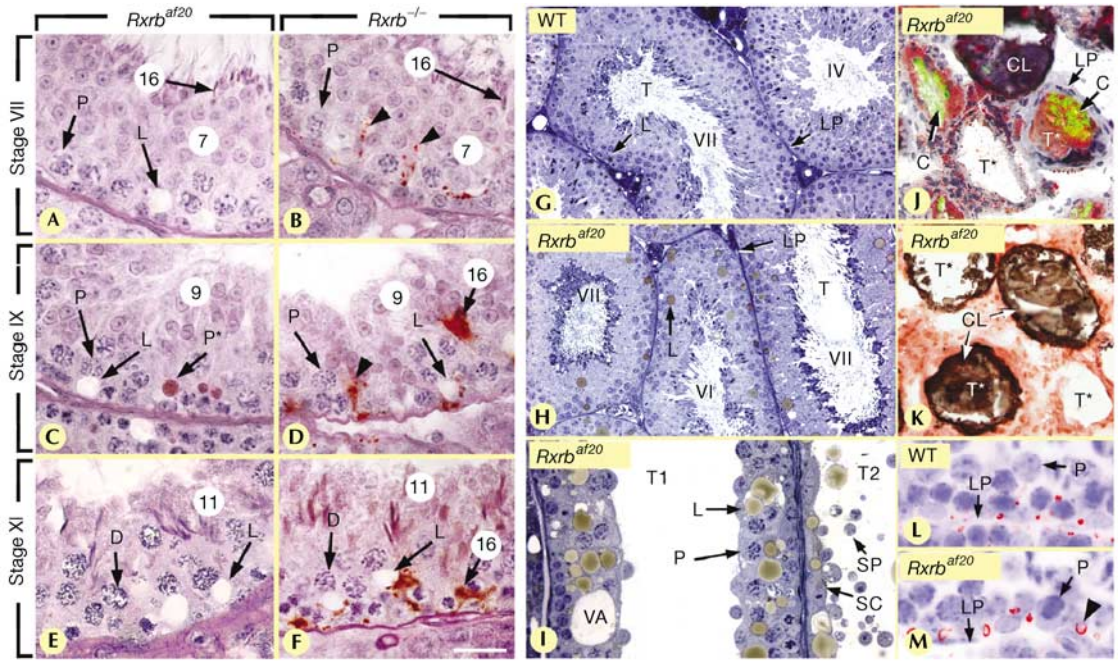

Fig 1 | Normal spermatid release, but progressive lipid accumulation and fatty degeneration in *Rxrb*^{af20} testes. Histological sections at 1 (**L,M**), 5 (**A–F**), 12 (**G–I**) and 24 (**J,K**) months of age. (**A–F**) TUNEL analysis and PAS staining prior to the onset of degeneration: in *Rxrb*$^{-/-}$ testes, mature spermatids (step 16) at an early phase of their retention are TUNEL-negative (**B**), but become positive (brown immunoperoxidase signals, arrowheads) upon uptake and degradation by SCs (**D,F**); basal apoptotic activity, reflected by the occurrence of TUNEL-positive spermatocytes (P* in (**C**)), is indistinguishable in *Rxrb*^{af20}, *Rxrb*$^{-/-}$ and WT testes. Note that 'holes' in the seminiferous epithelium correspond to lipid droplets (L) extracted during paraffin embedding. (**G–I**) Sections stained with osmium tetroxide and toluidine blue: (**H, I**) are from the same mutant testis that, apart from a majority of normal tubules (**H**), also contains tubules lacking spermatids (**I**; T1), or all meiotic or post-meiotic germ cells (T2). Large and empty vacuoles (VA) are probably accounted for by detachment from the epithelium of immature germ cells (SP). (**J**) Frozen unfixed histological section stained with oil red O and haematoxylin. Visible and polarized light views are superimposed. Lipids filling tubular ghosts are either in liquid form (oil red O stained) or in solid refringent crystals (false green colour). (**K**) Frozen section stained by the Von Kossa method and counterstained with safranin O. Calcified lipids stain brown. (**L, M**) Adipophilin forms ring-like figures around lipid droplets (red immunofluorescent signal, arrowhead); nuclei are stained with DAPI (blue). C, cholesterol crystals; CL, calcified lipid deposits; D, diplotene spermatocytes; L, lipid droplets; LP, lamina propria; P, pachytene spermatocyte; SC, Sertoli cell; SP, detached round spermatids; T, T1 and T2, seminiferous tubules; T*, tubular ghosts; VA, vacuoles. Numbers 7, 9, 11 and 16 refer to steps of spermatid maturation. Germ cell associations defining stages of the seminiferous epithelium cycle are indicated by roman numerals. Scale bar, 50 μm (**F**).

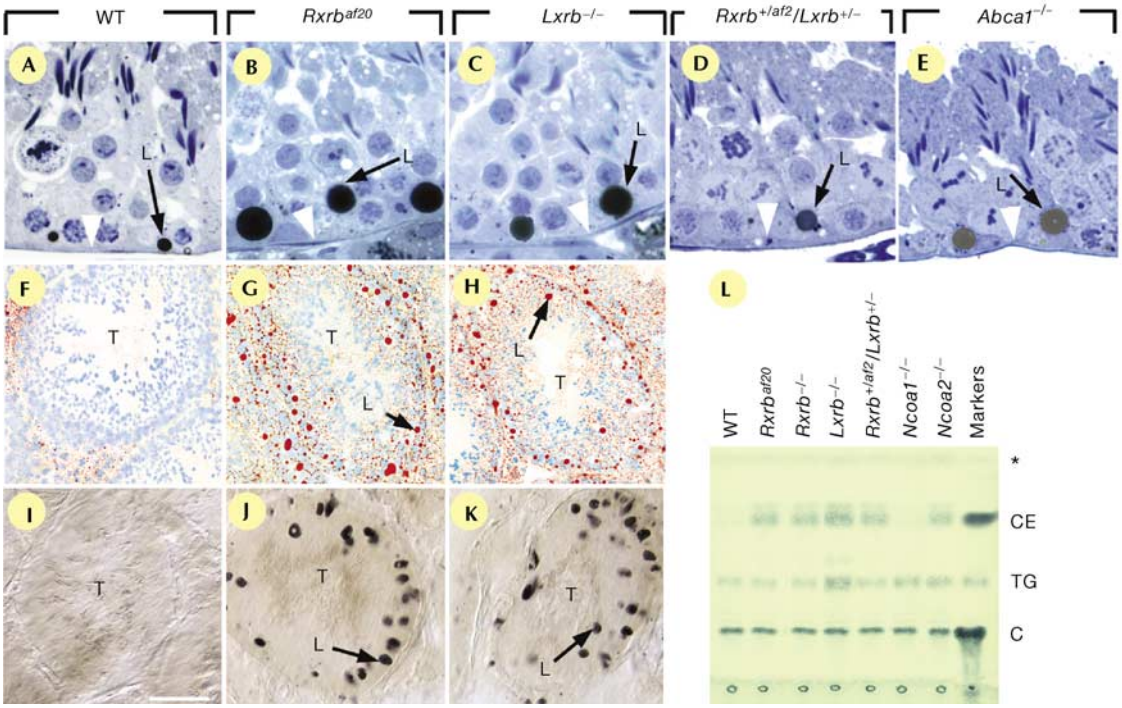

Fig 2 | Abnormal lipid droplets in *Rxrb*^{af20}, *Lxrb*$^{-/-}$ and *Abca1*$^{-/-}$ testes are indistinguishable and consist of cholesteryl esters. (**A–K**) Histological sections stained for lipids. (**A–E**) Osmium tetroxide blackens all unsaturated lipids; (**F–H**) the hydrophobic oil red O stains all neutral lipids including triglycerides and CE; (**I–K**) the Liebermann–Buchardt reaction is specific for cholesterol and its esters. Note that using these histochemical procedures, lipid accumulation was not detected in the liver and skeletal muscle of *Rxrb*^{af20} males at 24 ($n=3$) months of age. (**L**) Thin-layer chromatographs of lipids extracted from testes with the indicated genotypes. *Ncoa1* and *Ncoa2* are encoding SRC-1 and TIF2 coactivators, respectively. C, cholesterol; CE, cholesteryl ester (stearate); L, lipid droplets; T, seminiferous tubule; TG, triglyceride (triolein). The white arrowheads in (**A–E**) indicate the lamina propria, and the asterisk in (**L**) indicates the migration front. Scale bar, 50 μm (**I**).

increased phagocytosis in *Rxrb*^{af20} mice (Fig 1A,C,E). Normal phagocytosis of residual bodies by SCs displaying altered metabolic properties (see below) could contribute to the progressive enlargement of the lipid droplets over a period of several months. Becoming larger than nuclei, these droplets could mechanically impair SC cytoskeletal organization, thereby disrupting their adhesion to germ cells and, over time, kill SCs (Fig 1I). This mechanism probably represents the main, if not the only, cause of the seminiferous epithelium degeneration in *Rxrb*^{af20} mice. In any event, an RXRβ transcriptionally active AF-2 is essential to control SC lipid stores, whose increase leads to fatty degeneration.

Lipid overload in *Rxrb*^{af20}, Lxrb and *Ncoa2*-null mutants

We previously ascribed the lipid overload of *Rxrb*$^{-/-}$ SCs to triglyceride accumulation (Kastner *et al*, 1996). However, the crystals seen in degenerated *Rxrb*^{af20} testis (Fig 1J) suggested the presence of cholesteryl esters (CEs). That lipid droplets before degeneration were stained by oil red O and were positive in the Liebermann–Buchardt reaction confirmed the presence of CEs ($n=3$; Fig 2G,J). Additionally, compared to WT testes, thin-layer chromatography (TLC) analyses showed a large excess of CEs in lipid extracts from *Rxrb*^{af20} and *Rxrb*$^{-/-}$ testes ($n=3$), whereas triglyceride levels were not altered (Fig 2L). Thus, accumulation of CEs is responsible for lipid overload of both *Rxrb*^{af20} and *Rxrb*$^{-/-}$ SCs.

PPARα, β and γ, which require heterodimerization with RXR to function (Escher & Wahli, 2000), are involved in lipid and cholesterol homeostasis (Leibowitz *et al*, 2000; Chawla *et al*, 2001; Chinetti *et al*, 2001). Even though the α and β isotypes are expressed in SCs (Braissant *et al*, 1996), lipid accumulation was absent from the seminiferous epithelium of 12-month-old PPARα ($n=3$) and 22-month-old PPARβ ($n=3$) null mutants (data not shown). As PPARγ transcripts are not detected in SCs (Braissant *et al*, 1996), cholesterol homeostasis in SCs does not require PPAR.

LXRα and β, which are key regulators of cholesterol homeostasis, also heterodimerize with RXRs (Lu *et al*, 2001). Only the β isotype is expressed in the testis (Lu *et al*, 2001) with a transcript distribution pattern identical to that of RXRβ (i.e. in SCs and not in germ cells; data not shown). This organ was, however, not yet analysed as *Lxrb*$^{-/-}$ males were reported to be fertile (Alberti *et al*, 2001). Strikingly, 5-month-old *Lxrb*$^{-/-}$ testes ($n=2$; Fig 2C) were histologically identical to age-matched *Rxrb*^{af20} testes ($n=3$; Fig 2B) and their SCs displayed large lipid droplets containing CEs (Fig 2H,K,L). Most importantly, these large droplets were also observed in SCs of *Lxrb*$^{+/-}$/*Rxrb*$^{+/af2}$ compound heterozygotes ($n=3$; Fig 2D,L), but not in the corresponding single heterozygotes ($n=3$; data not shown). Thus, RXRβ/LXRβ heterodimers,

in which the RXRβ AF-2 is transcriptionally active, could be the functional units required for cholesterol homeostasis in SCs. Moreover, the observation that the large lipid droplets accumulating in SCs of mice lacking the TIF2 (*Ncoa2*$^{-/-}$) coactivator ($n=5$), but not in those lacking SRC-1 (*Ncoa1*$^{-/-}$; Xu *et al*, 1998; Gehin *et al*, 2002; data not shown), were also rich in CEs (Fig 2L) provides the genetic evidence that the TIF2 coactivator could specifically mediate the transcriptional activity of RXRβ/LXRβ heterodimers.

RXRβ/LXRβ heterodimers control cholesterol efflux in testis

We next investigated the expression of genes involved in cholesterol homeostasis. In peripheral cells (e.g. macrophages), when the amount of cholesterol exceeds the storage capacities, increased cholesterol efflux mediated by membrane ATP-binding cassette (ABC) transporters delivers cholesterol to high-density lipoprotein (HDL) in the serum for transport back to the liver (Dean *et al*, 2001). In this process of reverse cholesterol transport, RXRα/LXR heterodimers have key roles by directly controlling the expression of the ABCA1, ABCG1, ABCG5 and ABCG8 transporters, as well as that of apo-lipoprotein E required for HDL formation (Repa *et al*, 2000; Claudel *et al*, 2001; Laffitte *et al*, 2001; Lu *et al*, 2001). In *Rxrb*^{af20} testis, expression of *Abcg1*, *Abcg5*, *Abcg8* and *Apoe* was not significantly altered, but *Abca1* transcript levels were decreased by 30% (Fig 3A; data not shown). In *Lxrb*$^{-/-}$ testis, *Abca1* transcript levels were also decreased (by about 40%; Fig 3A), and the amounts of ABCA1 in protein extracts from both *Rxrb*^{af20} and *Lxrb*$^{-/-}$ testes were accordingly decreased

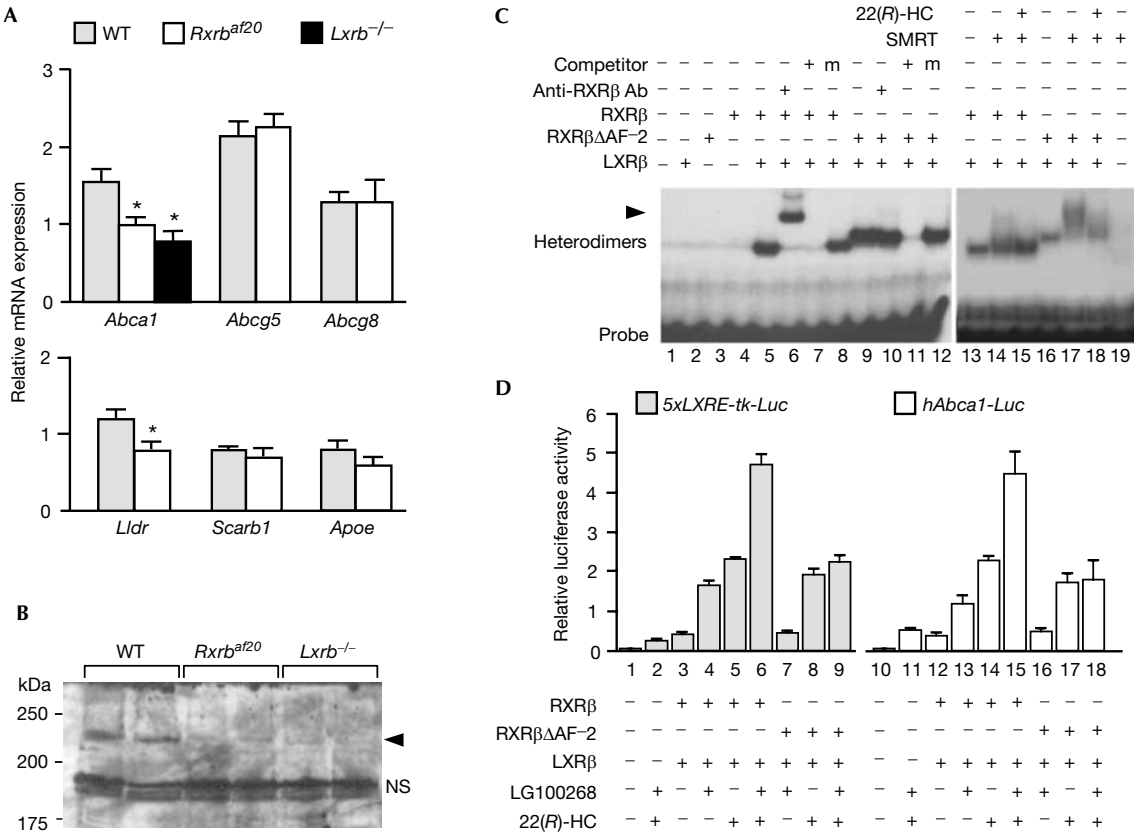

Fig 3 | Expression of genes involved in cholesterol homeostasis in the testis and transcriptional activity of RXRβ/LXRβ heterodimers. (**A**) Total RNA from WT (grey bars), *Rxrb*^{af20} (white bars) and *Lxrb*$^{-/-}$ (black bar) mice was analysed by quantitative RT-PCR. The ABC transporters (*Abca1*, *Abcg5* and *Abcg8*) are involved in cholesterol efflux. LDL receptor (*Ldlr*), scavenger receptor SR-B1 (*Scarb1*) and apo-lipoprotein E (*Apoe*) are involved in uptake and reverse transport of cholesterol. The bars represent the mean ± s.d. of the mRNA levels (standardized to GAPDH controls) in testes from five individuals of each genotype. The asterisks indicate significant differences ($P<0.05$). (**B**) Western blot analysis of proteins from WT, *Rxrb*^{af20} and *Lxrb*$^{-/-}$ testes ($n=2$) with an anti-ABCA1 polyclonal antibody, showing decreased levels of ABCA1 (arrowhead) in both mutants. The lower band is a nonspecific signal (NS) attesting similar loading on each lane. (**C**) EMSA showing that RXRβ/LXRβ (lanes 5 and 13) and RXRβΔAF-2/LXRβ (lanes 9 and 16) heterodimers bind to the *Abca1* LXRE (probe). Addition of an antibody against the RXRβ C-terminal region supershifted the complexes with RXRβ (lane 6) but, as expected, not with RXRβΔAF-2 (lane 10). Competitions were performed using a 1,000-fold excess of unlabelled LXRE (lanes 7 and 11) or mutated LXRE (m, lanes 8 and 12). Incubation in the presence of purified SMRT supershifted the complexes (lanes 14 and 17), while addition of 22(*R*)-HC inhibited the SMRT-induced supershift (lanes 15 and 18). (**D**) Cell transfections with either the *5xLXRE-tk-Luc* (grey bars) or the *ABCA1-Luc* (white bars) reporter, together with RXRβ, RXRβΔAF-2 and LXRβ expressing vectors, as indicated. Ligands for RXRβ (0.1 μM LG100268) and/or LXRβ (10 μM 22(*R*)-HC) were added as indicated. The bars show mean luciferase activity ± s.d. of triplicate transfections.

(Fig 3B). Importantly, LXRβ mRNA levels were not altered in *Rxrb*^{af20} testis (data not shown). It is therefore likely that RXRβ/LXRβ heterodimers control ABCA1 expression.

As deleting AF-2 helix 12 could convert an RXR to a transcriptional repressor due to increased affinity for co-repressors, including SMRT (Zhang *et al*, 1999), the *Rxrb af2* mutation may silence the RXRβΔAF-2/LXRβ heterodimer activity. Using electrophoretic mobility shift assays (EMSA) on the *Abca1* LXR-responsive element (LXRE), we first showed that deleting helix 12 of RXRβ did not impair its heterodimerization with LXRβ (Fig 3C). Interestingly, although RXRβΔAF-2 recruited SMRT more readily than RXRβ in these assays (compare lanes 14–17), adding an LXR ligand (22(*R*)-hydroxycholesterol (22(*R*)-HC)) strongly decreased SMRT binding (lane 18). Since LXRβ is probably oxysterol-liganded in SCs, which synthesized steroids (Ford *et al*, 1999), these data strongly suggest that the RXRβ AF-2 is crucial for *Abca1* transcription.

To further support this possibility, we analysed the activities of an LXRE-containing reporter and the *Abca1* promoter in transient transfection assays (Fig 3D). Compared to basal conditions (lanes 3 and 12), RXRβ/LXRβ-transfected cells treated either with the RXR-selective ligand LG100268 (lanes 4 and 13) or with 22(*R*)-HC (lanes 5 and 14) exhibited a four- and sixfold increased transcription, respectively. When both compounds were added together (lanes 6 and 15), there was a synergistic 12-fold induction. Importantly, in RXRβΔAF-2/LXRβ-transfected cells, transcription was still increased fivefold by 22(*R*)-HC (lanes 8, 9, 17 and 18), but no longer by LG100268 (lanes 7 and 16). Thus, both ligand-activated RXRβ and LXRβ effectively contribute to the transcription of the responsive genes, and deleting RXRβ AF-2 abrogates selectively the RXRβ-dependent activity. Therefore, assuming that an AF-2 helix 12 deletion is equivalent to the absence of an agonistic ligand (see Mascrez *et al*, 1998), a liganded RXRβ appears to be required to maintain ABCA1 expression to a level compatible with efficient cholesterol efflux from SCs. Interestingly, as CEs do not accumulate in vitamin A-deficient testes (Kastner *et al*, 1996), our study also indicates that this ligand is most probably not 9-*cis* retinoic acid.

In *Rxrb*^{af20} testes, expression of low-density lipoprotein (LDL) receptor (*Ldlr*), involved in cholesterol uptake, was decreased by 30%, whereas expression of HDL receptor (*Scarb1*), involved in reverse cholesterol transport and expression of HMG-CoA synthase (*Hmgcs1*) and reductase (*Hmgcr*), both involved in cholesterol synthesis, was not changed (Fig 3A; data not shown). Taken together, these data indicate that CE accumulation in SCs of *Rxrb*^{af20} mutants primarily results from a decreased cholesterol efflux, not compensated by a reduced LDLR-mediated uptake or synthesis rate. Along these lines, it is noteworthy that the seminiferous epithelium of *Abca1*$^{-/-}$ mutants ($n=2$) displays multiple vacuoles, which correspond to CE droplets indistinguishable from those observed in *Rxrb*^{af20} and *Lrxb*$^{-/-}$ testes (Fig 2E and data not shown; Christiansen-Weber *et al*, 2000).

In conclusion, our genetic and molecular data demonstrate that RXRβ/LXRβ heterodimers, in which the RXRβ AF-2 is transcriptionally active, control ABCA1 transporter-mediated cholesterol efflux in SCs, and also that, if RXRβ AF-2 activity requires the binding of a ligand, it is unlikely to be a retinoid. Our results suggest furthermore that altered RXRβ/LXRβ-dependent signalling pathways could be responsible for SC lipid overloads that are observed in human testes during senescence and in some azoospermic patients (Holstein *et al*, 1988). Lastly, with respect to RXR/LXR-dependent, ABCA1-mediated cholesterol efflux, it is interesting to note the parallel between SCs, which are endowed with potent phagocytic properties, and macrophages from the arterial wall (Claudel *et al*, 2001).

METHODS

Mice. All mice were on a mixed C57BL/6-129/Sv genetic background and housed in an animal facility licensed by the French Ministry of Agriculture (Agreement No. B67-218-5, 1999-02-09). Animal experiments were supervised by M.M. who is qualified for experimenting with mice.

Histology, immunohistochemistry and lipid analysis. Periodic Acid Schiff (PAS) reaction, osmium tetroxide and toluidine blue stainings, oil red O and TUNEL assays were performed as described (Kastner *et al*, 1996; Gehin *et al*, 2002). Cholesterol and CEs were detected using Romieu's modification of the Liebermann–Buchardt reaction, whereas calcified lipids were stained using Von Kossa's method (McManus & Mowry, 1965). The CE-rich adrenal gland and triglyceride-rich brown fats were used as positive and negative controls, respectively. Anti-mouse adipophilin antibodies (Research Diagnostic) diluted 1:100 were revealed using a Cy3-labelled secondary antibody at 1:200 dilution (Euromedex). Lipids from testes ($n=3$) were extracted using the Bligh and Dyer procedure, resuspended in 0.5 ml of methanol–chloroform (1:2, v/v), loaded onto TLC plates (Machery–Nagel) that were run in heptane–diethyl ether–acetic acid (7:2:1, v/v) for 5 min, then in heptane for 10 min, and revealed by molybdatophosphoric acid staining. TLC analyses were repeated three times. Standard lipids were from Sigma.

RNA and protein analyses. Deletion of region coding for the RXRβ AF-2 (helix 12) core was confirmed by sequencing reverse transcription polymerase chain reaction (RT-PCR) products amplified from *Rxrb*^{af20} E12.5 embryo RNA. Quantitative analysis of RNA was carried out by real-time RT-PCR using a Light-Cycler and the DNA double-strand-specific SYBR Green I dye for detection (Roche). Total RNA was reverse transcribed, and purified cDNAs were tested in duplicate by RT-PCR. Results were normalized to GAPDH levels (see supplementary information online). Cytosolic extracts (100 μg proteins from 2-month-old mice) were resolved on 7% SDS–PAGE and blotted onto nitrocellulose membranes (Millipore). ABCA1 was detected using a rabbit polyclonal antiserum diluted 1:50 (Abcam). Immunoreactions were visualized using protein A-coupled horseradish peroxidase (dilution 1:5,000), followed by chemiluminescence (Amersham).

EMSA and cell transfections. EMSA were performed on the mouse *Abca1* LXRE using *in vitro*-translated receptors, whereas transfections were carried out in Hek293 cells (see supplementary information online).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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